ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, SUPPLEMENT **1**, 223-231 (1962)

A Study of the Factors Influencing the Rate and Extent of Enzymic Reactivation during Reoxidation of Reduced Ribonuclease

CHARLES J. EPSTEIN, ROBERT F. GOLDBERGER, D. MICHAEL YOUNG, AND CHRISTIAN B. ANFINSEN

From the Laboratory of Cellular Physiology and Metabolism, National Heart Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Maryland

Received May 3, 1962

The rate and extent of return of enzymic activity during oxidation of the sulfhydryl groups of reduced ribonuclease were studied under a variety of conditions. The rate of reactivation was found to be inversely proportional to the concentration of reduced protein. Under the most favorable conditions employed in this study (0.01 mg. protein/ml., pH 8.2, 24° C.), enzymic activity was demonstrable within 5 min. and full restoration of activity was attained within 60 min. Studies of the sedimentation behavior of ribonuclease during the course of reoxidation indicated that intermolecular disulfide bond formation occurs early in the reoxidation process. In agreement with the hypothesis that the native configuration is the thermodynamically most stable one, the aggregated material subsequently undergoes a spontaneous conversion to the fully active monomer. These findings indicate that the marked delay in reactivation previously observed with more concentrated solutions of the enzyme is not an inherent feature of the reoxidation process, but is, rather, the result of a concentration dependent intermolecular aggregation.

Variations in pH and temperature were found to affect both the rate and the extent of enzymic reactivation, whereas orthophosphate ions, ribonucleic acid, native ribonuclease, and low concentrations of β-mercaptoethanol had no effect.

These findings are consistent with the hypothesis that the information necessary for the conversion of a newly synthesized polypeptide chain to the native form of the protein molecule resides in the amino acid sequence and does not involve a genetically determined template.

INTRODUCTION

It is now widely accepted that the linear sequence of amino acids in a protein molecule is determined by the sequence of nucleotides in a portion of the deoxyribonucleic acid (DNA) of the cell nucleus, and that a ribonucleic acid (RNA) "messenger" is the intermediate between DNA and protein. Studies of nonuniform labeling of hemoglobin (1, 2), egg white lysozyme (3), and the protein of *Escherichia coli* (4) have, furthermore, indicated that synthesis of a protein molecule starts at the NH_2-terminal end and proceeds in a linear fashion to the COOH-terminal end of the chain. Such chains must subsequently assume a unique "native" configuration stabilized by numerous side chain interactions, interpeptide-bond hydrogen bonds and disulfide bonds. A major gap in our understanding of protein synthesis involves the mechanism by which the newly formed chain of amino acids is converted to the folded configuration of the native protein. It has been suggested that there may be a genetically determined template for the folding process in addition to the template determining the amino acid sequence. A second possibility is that the amino acid sequence itself is the principal

determinant of the final three-dimensional structure, and that the protein assumes the structure having the lowest configurational free energy. This hypothesis does not exclude the possibility that there is a stepwise folding of the molecule, starting from one end of the polypeptide chain, or that substrate or some other complementary material may influence the folding process.

Previous work in this laboratory on bovine pancreatic ribonuclease (RNase) (5, 6) has demonstrated that after reduction of its four disulfide bridges and disruption of noncovalent linkages in β-mercaptoethanol and 8 *M* urea, RNase is capable of reoxidation to a form indistinguishable from the native enzyme. More recently, the partial or complete recovery of activity following reoxidation of reduced egg white lysozyme (7, 8), Taka-amylase A (7), insulin (9–11) and trypsin (12, 13) have been reported. Previous studies of the kinetics of reoxidation of RNase (14) showed that free sulfhydryl groups disappeared at a moderate rate (about 50% in 200 min.), paralleling a change in optical rotation from a value characteristic of reduced RNase to that characteristic of the native protein. Enzymic activity, however, did not appear until after 90–100 min. of incubation and did not reach 50% of native activity until after 400 min. It was postulated that the extended delay in the appearance of enzymic activity (previously termed the "lag phase") was due to an initial random formation of disulfide bonds followed by rearrangement, through disulfide interchange, to yield the correct pairing of half cystine residues. Indeed, preliminary peptide mapping of enzymic hydrolyzates indicated that during this period the protein does contain "incorrect" disulfide bonds (14).

The existence of a period during which no enzymic activity was observed and the relatively long time required for restoration of full activity presented difficulties in interpreting the significance of these experiments when considered in terms of the rapidity of polypeptide chain synthesis (2–4). The present paper summarizes further investigations of the kinetics of reactivation during reoxidation of reduced RNase. A study of the concentration dependence of this process suggests that the lag phase and the slow reactivation previously observed are not inherent properties of individual molecules, but are the result of molecular aggregation. Studies were also made of the effects produced by variations in temperature and in pH, and by the presence of sulfhydryl compounds, of RNA, of native RNase, and of orthophosphate ions.

EXPERIMENTAL

Materials and Methods

Reduction and Reoxidation of Ribonuclease

Five times recrystallized bovine pancreatic RNase (lot #R81B-249), was purchased from Sigma Chemical Company, St. Louis, Missouri. The enzyme was reduced by treatment with β-mercaptoethanol (Eastman, white label), in freshly prepared 8 *M* urea (recrystallized from 95% ethanol), at pH 8.2 for a period of 20 hr. (6). After reduction, the protein was separated from the reagents by passage through a column of Sephadex G-25 (Pharmacia, Uppsala), equilibrated with 0.1 *M* acetic acid. Titration of the reduced RNase with mercuribenzoate (15) demonstrated the presence of the expected number (8.0 ± 0.2) of sulfhydryl groups.

Unless otherwise specified, reoxidations were carried out in a total volume of 10 ml. in a 50-ml. Erlenmeyer flask, at a protein concentration of 0.02 mg./ml., at 24° C., and at a final pH of 8.2 in 0.09 *M* Tris–chloride buffer [reagent-grade tris(hydroxymethyl)aminomethane, Sigma Chemical Company].

Assay for Enzymic Activity

Enzymic activity was assayed by digestion of yeast RNA (16), followed by precipitation with uranyl acetate–perchloric acid solution (17). In those experiments in which the protein was reoxidized at a concentration of 0.02 mg./ml., aliquots containing 1, 2, 5, and 10 μg. of protein were assayed. In experiments in which the protein was reoxidized at higher or lower concentrations, aliquots of the same volume (containing proportionately larger or smaller amounts of protein) were assayed.

Velocity-Sedimentation Determinations

The sedimentation behavior of RNase during reoxidation was examined with the Spinco Model E ultracentrifuge, employing schlieren optics. In order to observe sedimentation as soon as possible after reaching 59,780 r.p.m., and to minimize boundary spreading, a double-sector, capillary-type synthetic boundary cell was employed. All runs were made close to 25° C., and rotor temperature was maintained (± 0.05° C.) by the RTIC unit supplied with the instrument. Photographic plates were analyzed with a Bausch and Lomb microcomparator equipped

with a specially constructed stage for vertical and horizontal alignment of the plate. All sedimentation coefficients were corrected to a standard state of water at 20° C. The partial specific volume of RNase was assumed to be 0.695 (18).

RESULTS

Effect of Concentration

The rate of return of enzymic activity was determined for RNase solutions reoxidized at protein concentrations ranging from 0.005 to 0.1 mg./ml. The findings are presented in Fig. 1. In all cases except 0.1 mg./ml., activity was detected in the earliest aliquots (5–10 min.), and the rate of return of enzymic activity was closely related to the concentration of protein during reoxidation. After incubation for 60 min. at the highest concentration (0.1 mg./ml.) only 11% of the final activity had returned, while at 0.01 and 0.02 mg./ml. the return of activity was complete in this time period. The data for 0.005 mg./ml. are not included in Fig. 1. In this case the initial rate of appearance of enzymic activity was greater than for 0.01 mg./ml., but the final level attained was less than 100%. The latter phenomenon may be due to significant adsorption of the enzyme to the glassware at the low protein concentration. Although the return of activity was usually complete in 60 min. at the 0.02 mg./ml. level, 100–150 min. was occasionally required.

Effect of Sulfhydryl-Containing Compounds

β-Mercaptoethanol was added to the reoxidation mixtures in amounts which gave molar ratios of added sulfhydryl groups to protein sulfhydryl groups ranging from 0.5:1 to 3000:1. The results are summarized in Fig. 2. No effect was noted at ratios between 0.5:1

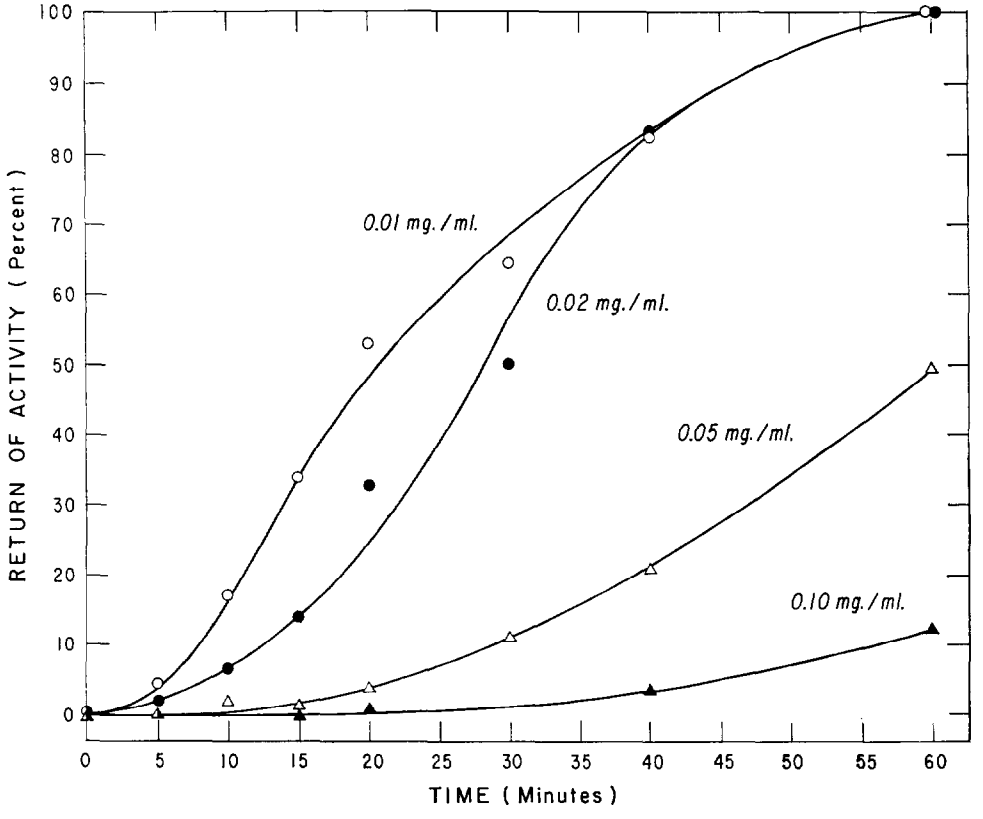

FIG. 1. Effect of protein concentration on rate of return of enzymic activity. Reduced RNase, at concentrations from 0.01 to 0.10 mg./ml. was reoxidized at pH 8.2 in 0.09 *M* Tris buffer, 24° C. Enzymic activity was determined as described in the text.

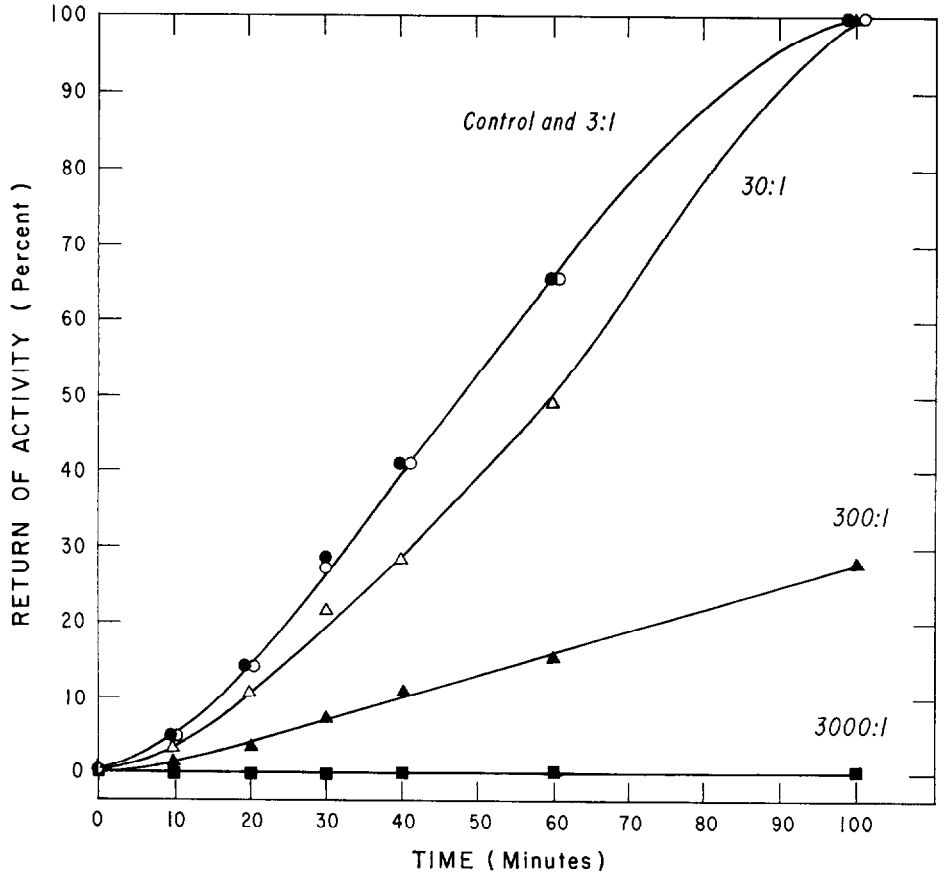

FIG. 2. Effect of β-mercaptoethanol on rate of return of enzymic activity. β-mercaptoethanol, at molar ratios to protein sulfhydryl groups of 3:1 to 3000:1, was added to a solution of reduced RNase (0.02 mg./ml.) in 0.09 *M* Tris buffer, pH 8.2, 24° C.

and 3:1. At higher ratios (30:1, 300:1), there was a decrease in the rate of reactivation, but not in the final level of enzymic activity. At a ratio of 3000:1, reoxidation, and hence return of activity, was completely inhibited over the time period of the experiment. Cysteine, in ratios of 3:1 and 300:1 produced a moderate reduction in the rate, but not in the extent of return of enzymic activity.

Kinetics of Reoxidation as Followed by Velocity-Sedimentation

Ribonuclease, at a final concentration of 1.0 mg./ml., was reoxidized at pH 8.2 and 24° C., in 0.2 *M* Tris–acetate buffer. Aliquots were removed at various times during the course of the reoxidation and were examined in the ultracentrifuge. A zero-time sample was run at pH 4.76, rather than 8.2, thus avoiding reoxidation of sulfhydryl groups, which proceeds extremely slowly at low pH values.

Sedimentation coefficients were calculated from a series of photographs taken every 4 min. after the rotor had reached full speed. In each case, only one boundary was observed, although considerable boundary spreading, indicating heterogeneity, was noted for the 100-min. sample. For this reason, the $S_{20,w}$ values represent weight-average sedimentation coefficients. Area analysis of the schlieren patterns at the beginning and end of each run demonstrated that no significant amount of protein escaped detection by sedimenting rapidly to the bottom of the cell. The data are summarized in Fig. 3. In the absence of β-mercaptoethanol, there was an initial rapid increase in $S_{20,w}$, followed by a decrease.

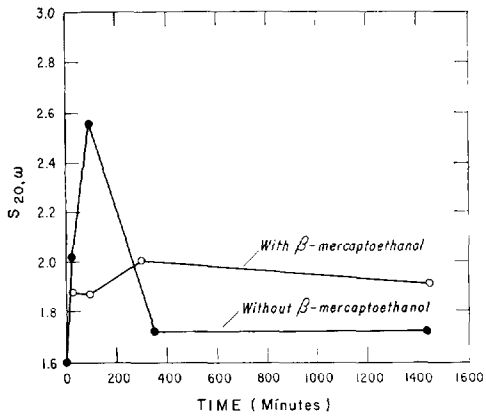

FIG. 3. Course of reoxidation as followed by velocity-sedimentation determinations. Reduced enzyme, at a final concentration of 1.0 mg./ml., was reoxidized in Tris–acetate buffer at pH 8.2, 24° C., in the presence and absence of β-mercaptoethanol. The molar ratio of mercaptoethanol to protein sulfhydryl groups was 150:1. $S_{20,w}$ is expressed in Svedberg units.

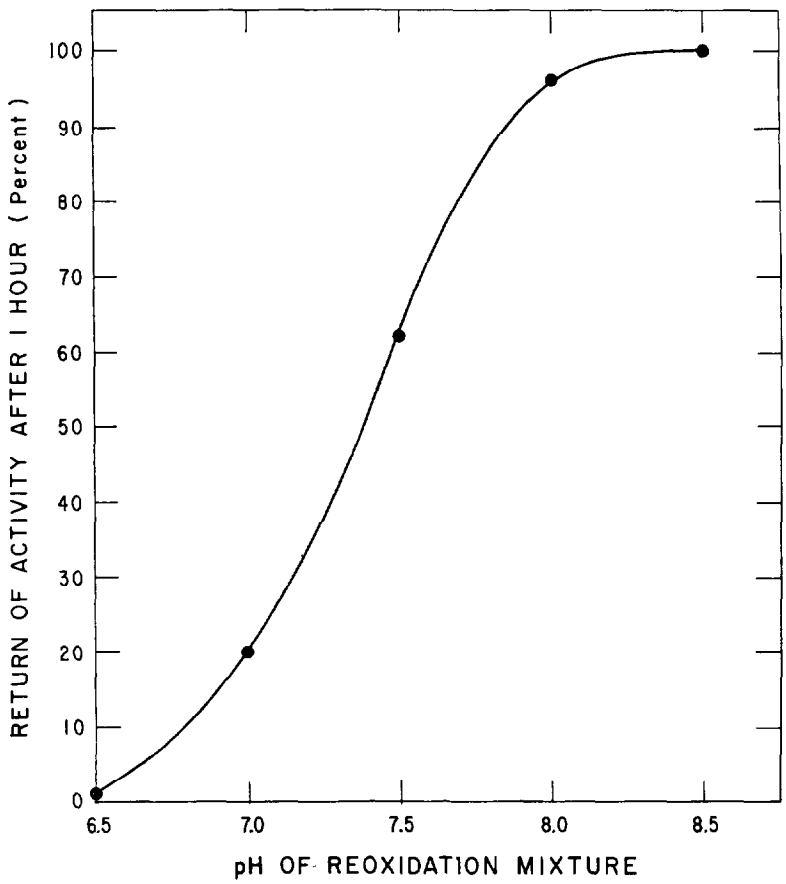

FIG. 4. Effect of pH on rate of return of enzymic activity. Reduced RNase, at a concentration of 0.02 mg./ml., was reoxidized in Tris buffer at pH values between 6.5 and 8.5. The per cent return of enzymic activity after 1 hr. is plotted as a function of pH.

These results, when compared with the values of $S_{20,w}$ at 1.0 mg./ml. for native and reduced carboxymethylated RNase, 1.89 *S* (18) and 1.72 *S* (19) respectively, indicate that there was initial molecular aggregation, followed by disaggregation as reoxidation proceeded and activity appeared. When reoxidation was carried out in the presence of β-mercaptoethanol, at a molar ratio of β-mercaptoethanol to enzyme sulfhydryl groups of 150:1, there was a marked reduction in the rate and extent of this aggregation process.

Effect of Variations in pH

Figure 4 shows the amount of reactivation obtained after 60 min. of reoxidation of reduced RNase at various pH values in 0.09 *M* Tris buffer. Reoxidation at pH 6.5 to 7.0 resulted in the appearance of only small amounts of enzymic activity, while at pH 8.0 to 8.5, 96–100% activity was regenerated.

Effect of Variations in Temperature

Reoxidation was carried out at three temperatures: 0.5, 24, and 37° C. As shown in Fig. 5, the rate of return of enzymic activity was most rapid at 24° and least rapid at 0.5°. The final extent of return of activity was less at both 0.5° (55%) and 37° (35%) than at 24°, at which temperature there was full reactivation.

Effect of Orthophosphate Ions

Orthophosphate ions are known to stabilize RNase against complete unfolding in the presence of urea (20). Because of this effect, experiments were performed to determine whether or not orthophosphate ions influence

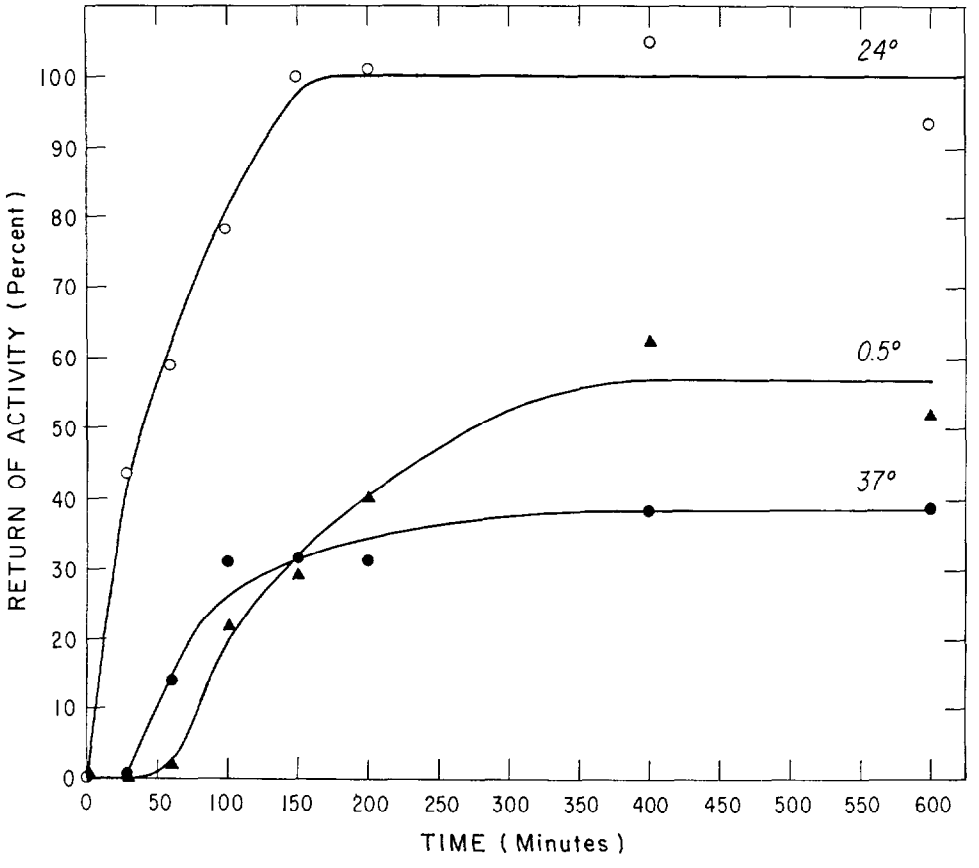

FIG. 5. Effect of temperature on the return of enzymic activity. Reduced RNase, at a concentration of 0.02 mg./ml., was reoxidized in Tris buffer, pH 8.2, at temperatures of 0.5, 24, and 37°C.

the return of enzymic activity during reoxidation of the reduced enzyme. There was no difference between the rates of return of activity in phosphate buffer and Tris buffer, both at pH 7.4.

Effect of Substrate

Reduced RNase was reoxidized in the presence and absence of RNA. The results are summarized in Table I. Approximately

TABLE I
EFFECT OF RNA ON RETURN OF ACTIVITY OF REDUCED RNASE

Time *min.*	Return of enzymic activity: Reoxidized in absence of RNA %	Return of enzymic activity: Reoxidized in presence of RNA %	Inhibition by RNA %
30	47	46	0
60	63	64	0
100	83	67	19
150	100	69	31
600	100	63	37

RNA, at a concentration of 0.04%, was added to a reoxidation mixture containing reduced RNase at a concentration of 0.02 mg./ml., in 0.09 *M* Tris buffer, pH 8.2, 24° C.

60% of the enzymic activity appeared at the same rate under both conditions, indicating that RNA does not affect the reoxidation process. However, gradually increasing inhibition of enzymic activity was noted thereafter in the material reoxidized in the presence of 0.05% RNA. This inhibition occurred only after a sufficient quantity of RNase had become active to digest a significant fraction of the RNA. The products of RNA cleavage, known to inhibit RNase (21), are probably responsible for the inhibition observed in the present study.

Effect of Native RNase

The kinetics of reappearance of enzymic activity during reoxidation of reduced RNase are consistent with an autocatalytic mechanism. To test this possibility, reduced RNase was reoxidized in the presence of native RNase added in an amount equal to 20% of the reduced enzyme. No increase in the rate of return of enzymic activity was effected by the presence of the native protein.

DISCUSSION

The data presented above demonstrate that the rate of return of enzymic activity during reoxidation of reduced RNase is inversely proportional to the concentration of protein in the reoxidation mixture. Maximal rates could not be measured because of technical difficulties at protein concentrations lower than 0.01 mg./ml. In previous studies (14), in which the protein concentration of the reoxidation mixture was 0.1 mg./ml., no enzymic activity was detected until after 75 min. of reoxidation. In the present study, in which larger aliquots of enzyme were taken for assay, activity at this protein concentration was detected after 20 min. (but not earlier). Only 10% of final activity had returned after 60 min. Allowing for differences in assay techniques, these findings are consistent with those of the previous study (14).

At a protein concentration of 0.05 mg./ml., activity was first detected after 10 min., while at concentrations of 0.02 mg./ml. and lower there was detectable activity within 5 min., the shortest time tested. At the latter concentrations, the half time for the return of enzymic activity was approximately 15–30 min. At all concentrations, the curves describing the return of enzymic activity during reoxidation of reduced RNase were sigmoid in shape, and the length of time required to reach the maximal rate of reactivation was proportional to the concentration of enzyme. These observations strongly suggest that the rate of return of enzymic activity is greatly affected by a concentration-dependent molecular aggregation. This conclusion is directly supported by the sedimentation data, which demonstrate marked aggregation early in the reoxidation process, followed by disaggregation. The period during which larger aggregates were found corresponds to the lag phase previously observed during reoxidation at a comparable protein concentration (14). In order to assess the relative importance of the two different kinds of aggregation that might be occurring (i.e., noncovalent interaction and intermolecular disulfide bonding), a similar ultracentrifuge experiment was carried out with reduced RNase in the presence of β-mercaptoethanol. The amount of β-mercaptoethanol employed in this experiment was

sufficient to slow the rate, without affecting the final extent, of regeneration of enzymic activity. The marked aggregation observed in the absence of β-mercaptoethanol did not occur under these conditions. The results of these experiments indicate that intermolecular disulfide bond formation is primarily responsible for the aggregation observed during reoxidation at a protein concentration of 1.0 mg./ml. Noncovalent interaction, if it occurs at all, appears to be only a minor factor in the aggregation process. These conclusions are consistent with the previously observed rapid disappearance of free sulfhydryl groups, the slow appearance of enzymic activity during reoxidation, and the occurrence of incorrect pairing of half cystine residues early in the reoxidation process (14).

Further studies of the effect of β-mercaptoethanol on the rate of return of enzymic activity showed that inhibition was proportional to the concentration of the reagent, and that a molar ratio of β-mercaptoethanol to enzyme sulfhydryl groups considerably greater than 300:1 was required to prevent reoxidation. At ratios between 0.5:1 and 3:1, β-mercaptoethanol had no effect on the rate of reactivation, although at these low concentrations it might have been expected to have accelerated the process by promoting disulfide interchange (22, 23). It should be noted, however, that the question of whether or not the molecule goes through a series of structures with incorrect pairing of half cystine residues before assuming the native configuration is not answered by these observations.

On the basis of the data presented above, it is concluded that reoxidation of reduced RNase under nonoptimal conditions involves a rapid polymerization, primarily through the formation of intermolecular disulfide bonds, followed by depolymerization, to yield the native protein with the correct pairing of half-cystine residues. At low concentrations of reduced RNase, intermolecular interactions are less probable and intramolecular disulfide bonding predominates. Variations in pH and temperature may alter the rate and extent of reactivation by affecting any of these processes, but it would be speculative, at this time, to assign mechanisms to these effects. However, it should be noted that the pH effect on the *rate* of return of activity is quite different from that observed by Haber and Anfinsen (24), who measured the final *extent* of return of activity at a higher protein concentration. In those studies, the pH dependence curve closely approximated the titration curve of the histidyl residues in a polypeptide chain, but no such relationship was observed in the present experiments.

No increase in the rate of reoxidation of RNase was effected by orthophosphate ions or RNA, substances which appear to stabilize the structure of RNase against unfolding in urea solutions (25). Similarly, native RNase did not alter the rate. These findings suggest that the substances tested do not influence the reoxidation process.

As was pointed out in the introduction, the reoxidation of reduced RNase has been employed as a model system to test the hypothesis that the final stage of protein synthesis, the folding of the newly formed polypeptide chain, does not involve specific genetic direction. All of the data presented are consistent with this hypothesis. However, these data also serve to remind us that the model is an imperfect one, and that *in vitro* conditions are undoubtedly very different from those *in vivo*. Nonetheless, in spite of the marked deviations of the *in vitro* conditions from the physiological, return of enzymic activity of reduced RNase begins within a few minutes and is complete within 60 min.

ACKNOWLEDGMENT

The authors wish to thank Mr. Jamie E. Godfrey for his technical assistance.

NOTE ADDED IN PROOF

Levinthal *et al.* (26), in a study of the reactivation of reduced *E. coli* alkaline phosphatase, have observed concentration and temperature dependences similar to those reported in this paper. However, β-mercaptoethanol was required in order to obtain good yields of reactivated alkaline phosphatase.

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